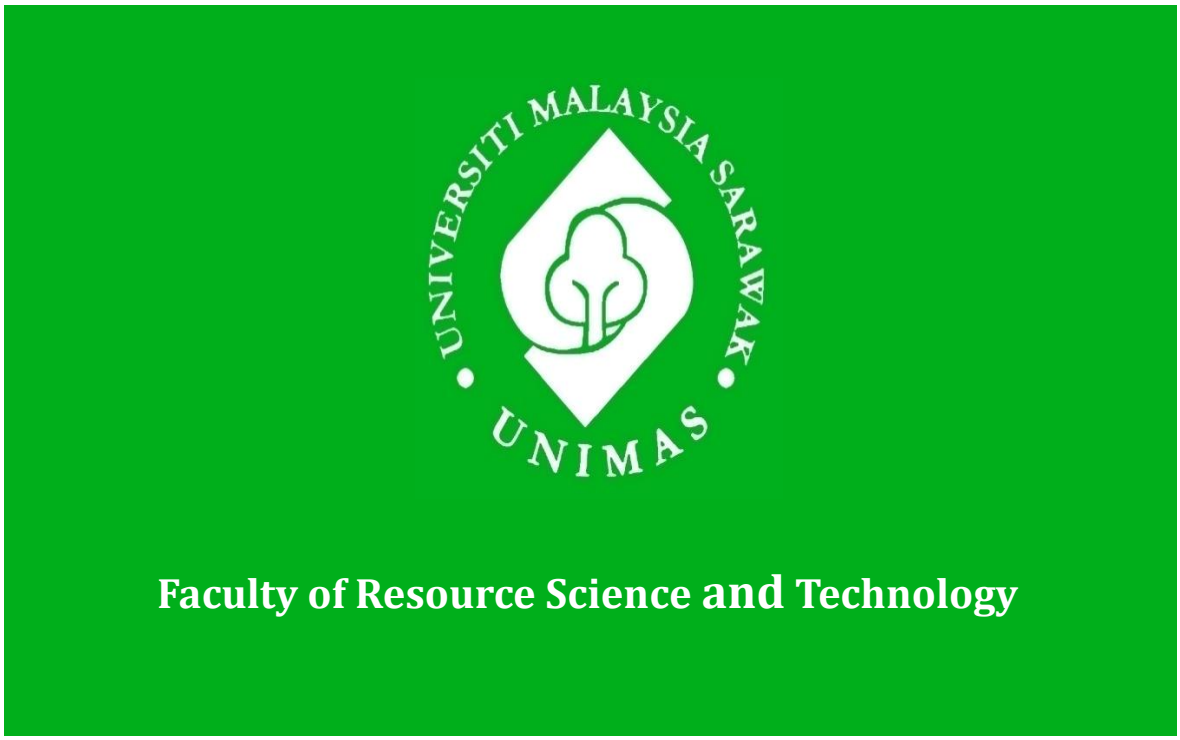

Population Genetics of *Tor douronensis* in Sarawak

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Bachelor of Science with Honours
Aquatic Resource Science and Management
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A report submitted in partial fulfilment of the Final Year Project STF 3012

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DECLARATION

No portion of the work referred in this dissertation has been submitted in support of an application for another degree qualification of this or any other university or institution of higher learning.

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List of Abbreviations

Abbreviations	Description
CO1	Cytochrome oxidase 1
DNA	Deoxyribonucleic Acid
mtDNA	Mitochondrial DNA
%	Percentage
EDTA	Ethylene diaminetetra-acetic acid
CTAB	Cetyl-trimethylammonium bromide
CIA	Chloroform Isomyl Alcohol
ml	Mililiter
g	Gram
sdH_2O	Sterilized distilled water
µl	Micro liter
rpm	Rotation per minute
^oC	Degree celcius
UV	Ultraviolet
PCR	Polymerase Chain Reaction
µM	Micro molar
EtBr	Ethidium Bromide

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ABSTRACT

Tor douronensis, known locally as Semah, is one of the valuable and potential resources in Sarawak due to its high food value and demand as game fish. Several molecular studies related to *T. douronensis* had been carried out but limited in populations. No study had involved *T. douronensis* from Bakun. Therefore, this study was designed to infer population subdivision, the genetic structure, evolutionary neutrality, and population expansion of *T. douronensis* among 4 populations from Sarawak including Bakun using partial DNA sequencing of the Cytochrome c oxidase I (COI) mtDNA gene. A total 465 bp of CO1 gene of *T. douronensis* had been successfully amplified, and based on phylogenetic tree, there are 3 distinct geographical population subdivision observed (Central, Southern, and Northern population); 1st clade (haplogroup I) from Bakun, 2nd clade (haplogroup II) from Layar and 3rd clade (haplogroup III) from Ba Kelalan and Ulu Limbang. Overall, there were 13 haplotypes and none is shared by any population. Low level gene flow has been observed. Small number of migrants per generation ($Nm<1.0$) among the population indicated the small population or separated populations with large geographical and topological barrier. Population expansion was undergone for the species for the whole populations except for northern population as shown by small and non-significant values of the sum of the standard deviation of the observed ($SSD<0.5$) and expected mismatch distributions (unimodal) and Harpending raggedness index ($r <1.2$). Furthermore a large negative value and significant test of Fu' Fs in Bakun population suggested recent expansion. The result also suggested that all the populations do not deviate with evolutionary neutrality supported with no significant in Tajima's neutrality test ($p<0.05$).

Key words: *Tor douronensis*, COI gene, Population expansion, Population subdivision, evolutionary neutrality.

ABSTRAK

Tor douronensis dikenali sebagai Semah oleh penduduk tempatan merupakan salah satu sumber bernilai dan berpotensi di Sarawak kerana nilai makanan dan permintaan dalam sukan pancing. Beberapa kajian molecular telah dilakukan berkaitan spesis ini namun hanya melibatkan beberapa populasi. Tiada kajian lagi dilakukan untuk populasi Bakun. Justeru, kajian ini bertujuan untuk melihat pecahan populasi, struktur genetik, kebiasaan genetik dan pengembangan populasi Semah di Antara empat populasi Semah temasuk Bakun di Sarawak dengan menggunakan rantaian CO1 gene. Sebanyak 465 bp rantaian CO1 gene berjaya dianalisa, dan berdasarkan peta phylogenetic, terdapat 3 pecahan kumpulkan populasi dikenal pasti iaitu, Kumpulan I daripada Bakun, Kumpulan II daripada Layar dan Kumpulan III daripada Ulu Limbang dan Ba Kelalan. Terdapat 13 haplotype secara keseluruhan tanpa ada percampuran haplotype dalam setiap populasi.Tahap pengaliran gen yang rendah dapat diperhatikan dengan jumlah migrasi rendah ($Nm <1.0$) menunjukkan populasi yang kecil atau populasi tersebit terpisah dengan keadaan geogafi yang besar. Pengembangan populasi berlaku untuk semua populasi kecuali untuk populasi Ba Kelalan- Limbang dengan kadar kecil dan tidak signifikan kadar taburan kaitan ($SSD<0.5$), jangkaan mismatch (unimodal) dan nilai pengembangan Harpending ($r<1.2$). Kadar negatif yang diihat pada populasi Bakun menunjukkan terdapat pengembang populasi. Ujian kebiasaan Tajima menunjukkan semua populasi tidak menyimpang daripada evolusi neutraliti ($p<0.05$).

Kata kunci: *Tor douronensis*, COI gen, pengembangan populasi, pecahan populasi, evolusi neutraliti.

1.0 Introduction

Tor douronensis Valenciennes (1842) are one of the members of mahseer groups from the genus *Tor* Gray in the family Cyprinidae. It is one of the most important freshwater fishes in Malaysia (Mohsin and Ambak 1983; Roberts 1989; Litis *et al.,* 1997; Ng, 2004). It can be found in Peninsular Malaysia, Sarawak and Sabah. It is reported to inhabit the upper streams and headwaters of most major river systems (Kottelat *et al*., 1993 ; Rainboth, 1996). In Sarawak, *T. douronensis* locally known as Semah and this specie has high economic value to the local people.

The market price for mahseer is one of the highest due to their great taste, for example, the price of *T. douronensis* can reach above RM45/kg in the open market in Kapit, Sarawak (Ingram *et al.,* 2005). During field visit to Bakun Dam on April 2013, local people claimed that 'Semah' could is sold in Kapit wet market with price approximately between RM60/kg to RM100/kg. Thus, fishes of the genus *Tor* have great potential for freshwater aquaculture industry (Ingram *et al*., 2005). In addition, the *Tor* fishes are also recognized as an excellent game fish, and have high demand in the ornamental fish industry due to their attractive colors (Ng, 2004).

However, populations of these species are declining due to degrading environmental conditions by deforestation, logging and development of hydropower dam that may have disturb their natural habitat (Ng, 2004). Uncontrolled fish harvest (overfishing) due to its high price has also contributed to the reduction of their population size (Ng, 2004). To date, their distributions in Malaysian Borneo are now limited to the upper streams and protected areas of Sarawak and Sabah (Litis *et al*., 1997 ; Nyanti *et al*., 1999; Ng, 2004).

Although currently not listed by the IUCN as a protected or endangered species, the drastic decline in natural populations of *T. douronensis* has increased awareness among relevant authorities (e.g., Fisheries Department, Malaysia and policy makers) of the importance of the conservation and proper management of this species. Due to its economic importance, high commercial, recreational and conservational value, *T. douronensis* needs a proper management plan to ensure its sustainability. It is crucial to understand their taxanomy status, population distribution, genetic variability, levels of gene flow and populations subdivisions and for understanding factors contributing to fitness of *T. douronensis* so that more effective management plan could be developed with the aim to warrant the long-term maintenance of genetic diversity of cultured stocks, as well as to minimize potential adverse effects on the genetic integrity of the wild populations through proper stock enhancement practices.

In addition, *Esa et al.* (2008) had carried out *T. douronensis* studies involving Layar, Ba Kelalan and Ulu Limbang populations. However, he suggested that larger samples size per population and samples from other areas are required to reveal the actual genetic variation at the inter-population and intra-populations levels. In this study, sample from Bakun Dam have been included.

Thus, the aim of this study is to infer population subdivision, the genetic structure, evolutionary neutrality, and population expansion of *T. douronensis* among 4 populations from Sarawak using partial DNA sequencing of the Cytochrome c oxidase I (COI) mtDNA gene.

2.0 Literature review

2.1 The Cyprinids

Cyprinids are ray-finned teleosts (Class *Actinopterygii*) from infraclass *Teleostei* and the order *Cypriniformes.*The family *Cyprinidae* is the most diverse family of freshwater fishes in the world, with at least 210 genera and over 2000 species (Nelson, 1994). The typical features of the cyprinid body are the toothless jaw, pharyngeal teeth in the throat (ideal for characterization), the presence of the Weberian organ at the beginning of the vertebrate column, and complete lack of spiny rays on the dorsal fin (Orban and Wu, 2008). The cyprinids species occur naturally in wide range of habitats ranging from northern temperate zone to the tropical regions as well. It well adapted worldwide, absent only from South America, Australia and Antarctica (Mayden, 1991) and contain many culturally and economically important species.

2.2 The Southeast Cyprinids

The Southeast Asian region, which includes Peninsular Malaysia and the island of Borneo, has one of the highest diversity of freshwater fishes in the world (Zakaria Ismail, 1990). According to Fish Base, more than 600 species of freshwater fish are recorded in Malaysia.In Peninsular Malaysia, about 400 species have been described (Mohsin & Ambak, 1983), while more than 350 species of freshwater fishes have been recorded in Borneo (Inger & Chin, 1962; Roberts, 1989; Kottelat *et al*., 1993). The Family Cyprinidae forms the largest family in terms of number of genera and species, and it dominates almost every water body in the region (Mohsin & Ambak, 1983; Zakaria Ismail, 1990). Among the cyprinids, fishes of the genus *Tor*

Gray (subfamily Cyprininae), commonly known as the Mahseers, are one of the most important freshwater fishes (Mohsin and Ambak, 1983; Roberts 1989; Ltias *et al.*, 1997; Ng, 2004).

2.3 The Genus *Tor*

The Genus *Tor* Gray currently has 17 describe species from all across Asia (Ng, 2004). They are distributed throughout the Southern China, Indian subcontinent and Southeast Asia by inhabit the upper stream river system and headwaters of most major river system (Kottelat *et al.*, 1993; Rainboth, 1996). However, due to developments and over fishing within their natural habitat range, many *Tor* species critical habitats and spawning grounds have been disturbed (Esa *et al.,* 2008). Eventually some species have become endangered, for example *T. putitora* Hamilton and *T. tor* Hamilton are listed as endangered in West Bengal, India (Mijkherjee *et al.,* 2002).

There are only three common species reported living in Malaysia namely, *T. tambroides* Bleeker, *T. tambra* Valenciennes, and *T. douronensis* Valenciennes characterized by the presence of the median lobe (Kottelat *et al.,* 1993; Kottelat and Whitten, 1996; Ng, 2004; Esa *et al.,* 2008) . The taxonomic and systematic diagnosis of *Tor* is chaotic and scarce. *T. tambroides* and *T. douronensis* were considered as two valid species (Ng, 2004; Esa *et al.,* 2008). However, Roberts (1999) considered them as a single species and *T. tambra* as their junior synonym of their congener. The taxonomic status of *T. soro* Valenciennes had been revised and it is currently re-classified as *Neolissochilus stracheyi* due to absence of median

lobe (Rainboth, 1996). However, Kottelat (2000) suggested *T. soro* as a valid species but Lim *et al.* (1990) and Robert (1993) reported it as a junior synonym of *T. tambra.*

2.4 The *T. douronensis* Valenciennes (1842)

In East Malaysia, the *T. douronensis* is considered as dominant and widespread mahseer species in Sarawak compare to *T. tambroides* Bleeker (Esa *et al.*, 2006) Currently, in Sabah, only *T. douronensis* have been reported species found (Inger & Chin, 2002; Esa *et al*, 2006). In Sarawak, *T. douronensis* locally known as Semah. In term of characteristic, *T. douronensis* and *T. tambroides* morphologically difference are separated by the present of short median lobe in *T. douronensis* apart from long median lobe in *T. tambroides* (Ng, 2004).

To date, their population and natural habitat can be found in most of Sarawak river system such as Batang Ai River, Layar River, Spak River, Ulu Baleh River, Bario River, Kelalan River, and Ulu Limbang River (Ng, 2004; Esa *et al.*, 2006; Esa *et al.*,2008). *T. douronensis* prefer upper streams, fast flowing and clears river with stony, pebbly or rock bottoms (Ng, 2004). Thus, this species is sensitive with environmental changes. *T. douronensis* natural diet mainly plan base diet including leaves, flowers and fruits (Ng, 2004)

In Sarawak, *T. douronensis* is considered as highly valuable mahseer due to its potential for aquaculture industry (Ingram *et al.*, 2005), high market value due to their great taste (Esa *et al.*, 2011). In the open market in Kapit, the market price of standard size of *T. douronensis* is up to RM 100/kg (Esa *et al.*, 2011). Furthermore, due to its fighting strength, *T. douronensis* recognized as perfect game fish for angler to challenge and the demand of these fish is significantly increase as ornamental fish as their colorations is attractive (Ng, 2004).

For the past few decades, habitat destruction due to environmental degradation such as river pollution, deforestation watershed erosion and development increase the vulnerability of these species (Esa *et al.,* 2006). Overfishing also gave such impact for the species by greatly reducing the population size. It caused their distributions in most habitat now limited to the upper streams and protected area (Litis *et al.*, 1997; Nyanti *et al.*, 1999; Ng, 2004; Esa *et al.*, 2006).

2.5 Molecular studies on Cyprinidae

Since the economic importance along with declining population size, limited distribution and habitat degradation, the understanding of population structure and genetic variation of these species is needed for effective management and future conservation strategies (Esa *et al.*, 2011). The molecular approach also provide a better insight of overall population status and the genetic conservation management plan for long term maintenance of genetic diversity of cultured stock as well as wild populations (Nguyen *et al.*, 2006; Esa *et al.*, 2008).

Molecular approach has many advantage as more reliable, precise and consistent compare to the conventional systematic characters studies of species (Ryan & Esa, 2006). To date, the conventional morphological studies always reflected to the species habitat, environment and non-genetic factors (Vrijenhoek, 1998). Molecular technique is also the crucial technique particularly in defined the systematic status of endangered and protected species since the application of non-destructive sampling (Esa *et al.*, 2008). Only small quantity of sample DNA is needed for analysis and the tissue sample can be any tissue in the organisms.

For the past few decade, molecular approach studies had revealed the systematic and population structure of Family Cyprinidae. Among molecular phylogenetic studies of Cyprinidae include those by Briolay *et al.* (1998) on the cyprinids of Central and South America using cytochrome b (*cyt* b) sequences; and Liu and Chen (2003) on the East Asian cyprinids using control region (D-loop). Only one molecular phylogenetic study has been reported on the cyprinids of Southeast Asia which by Esa *et al.,* (2012). The important finding of the study is the monophyletic status between genus *Tor* and *Neolissochillus* thus further erected the reclassification of *Neolissochillus stracheyi* from genus *Tor*. The result also showed that the genus *Barbus* was the closest taxa to the genus *Tor*. Another interesting finding was the *B. gonionotus* was phylogenetically distinct from its morphologically similar species. *Barbonymus schwanenfeldii* (K2P distinct value= 15.1%) and did not group together in a single *Barbonymus* clade.

On the studies of Mahseer group, Ryan and Esa (2006) examined the phylogenetic relationship among the freshwater fishes of the genus *Hampala* in Malaysia using partial *cyt* b sequences and Nguyen *et al.* (2008) recently examined the phylogenetic relationship of selected mahseer throughout Asia using three mitochondrial DNA gene regions (16S rRNA, *cyt b* and ATPase 6-8). There are also studies of molecular systematic among mahseer in Malaysia using cytochrome oxidase I (COI) gene (Esa *et al.,* 2006), mitochondrial DNA diversity of *Tor douronensis* in Malaysia Borneo (Esa *et al.,* 2008) and genetic characterization of two mahseer using microsatellite markers from other cyprinids (Esa *et al.,* 2011). The common result for those studies is the monophyletic status between *T. douronensis* and *T. tambroides* which further reinforced their taxonomic status as distinct species. The studies also showed that the *T. douronensis* is divided into three different major group, Sabah group, North

Sarawak group and South Sarawak group. Meanwhile, there is different result from studies made by Esa *et al.* (2006) with more recent study made by Nguyen *et al.* (2008). Esa *et al.* (2006) stated that there is high level of intra and inter-population variations in *T. douronensis* while Nguyen *et al.* (2008) study showed that the level of intra-population variations of *T. douronensis* is low while it inter-population variations is high. The study also support that little or no migration occurred among the extant population separated by large geographic distances or river systems.

2.6 Mitochondrial DNA

Mitochondrial DNA (mtDNA) is the genetic material that exists outside the nucleus in eukaryotic cells (Zhang *et al.*, 2008). Mitochondria is a unique organelle in the cell because contain their own DNA. In the typical vertebrate, the size of the mitochondrial genome is approximately 16 kb and contained a set of 37 genes that encode two ribosomal RNAs, 22 transfer RNAs and 13 proteins (Hartmann, 2011). It also contained two non-coding regions, displacement loop and the light strand replication origin, which are involved in replication and transcription of the mitochondrial DNA (Hartmann, 2011).

The mitochondrial DNA (mtDNA) is usually preferred in molecular studies due to a few of major features of it such as maternally inherited, haploid single molecule and the entire genome is transcribed as a unit (Avise, 1994). Moreover, mtDNA is more stable and occurs in a much higher number of copies than nuclear DNA (Avise, 1994).

COI mtDNA gene is broadly used in analysis of phylogenetic and population structure analysis due to faster evolutionary rate compared to the 16S rRNA (Simon *et al*., 1994), and thus capable of providing a better resolution at the interspecific level. The amplification

primers usually used is oligonucleotide primers COIf (5'-CCT GCA GGA GGA GGA GAY CC-3', forward) and COIe (5'-CCA GAG ATT AGA GGG AAT CAG TG-3', reverse) (Palumbi *et al.*, 1991)

3.0 Materials and methods

3.1 Collection of samples

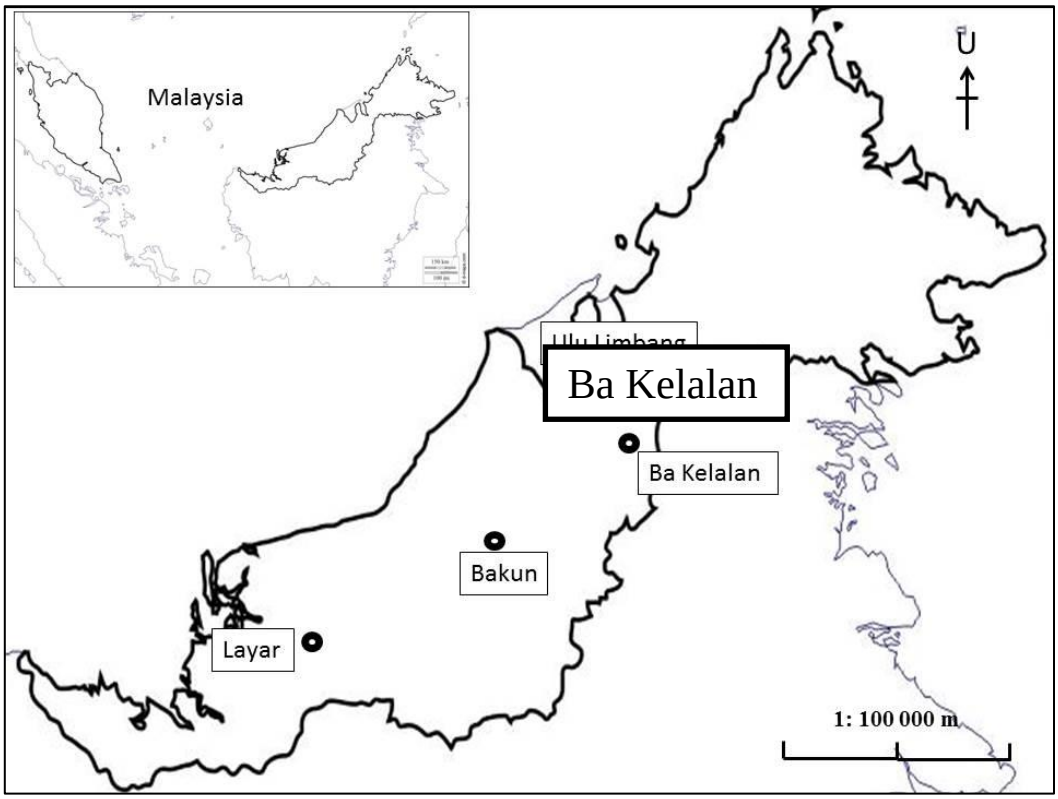

Figure 3.1 Location of *T. douronensis* populations involved in this study.

The total 11 sample of *T. douronenis* samples was obtained from Bakun Dam (N 02° 45′ 23″, E 114° 03′ 47″). Another samples were obtained from gene bank with accession number of (EF192444, EF192445) for Ulu Limbang samples, (EF192453, EF192454, EF192455, EF192456) for Layar samples and (EF192446, EF192447) for Ba Kelalan samples. Esa *et al.* (2008) reported both Ulu Limbang, Layar/Spak and Ba Kelalan river are only among few rivers left that have the *T. douronenis* populations. While in Bakun Dam, there is no reported study in population genetic aspects of *T. douronensis*. All samples were collected using

combination of methods namely cast net, and pole nets. The tissue, fin and scale from each sample was collected and the samples was preserved in 70% ethanol with 5% EDTA. The samples were identified using morphological data as in keys provided by Mohsin & Ambak (1983), Kottelat *et al.* (1993) and Inger & Chin (2002).

3.2 Total Genomic DNA extraction.

3.2.1 Preparation of buffer solution for modified CTAB method (Doyle and Doyle, 1987)

3.2.1.1 CIA Chloroform Isomyl Alcohol

For the preparation of 250 ml CIA, 240 ml of chloroform was mixed with 10 ml of isomyl alcohol and was stored at room temperature. The prepared CIA reagent was stored in flask (with appropriate labels), and wrapped with aluminium foil to avoid light for long term usage.

3.2.1.2 CTAB (Cetyl-trimethyl Ammonium Bromide)

Firstly, an amount of 40.90 g of NaCl was weighed using electronic balance (AR3130 Adventure™, Ohaus Corp.) and later placed in a 500 ml beaker. Then, 6.05 g of Tris base, followed by 10.00g of CTAB and 3.70 g of EDTA was added into the beaker. After that, 400 ml of sterilized distilled water, sdH_2O was poured into the beaker to dissolve the substances. Then, the solution was stirred using the magnetic stirrer until the solution become crystal clear for about 3 hours on a hotplate stirrer (Lab Tech® LMS-1003), however, the solution was avoided from evaporating. After the solution become clear, it let to cool down to room temperature. Later, the solution was poured into 500 ml sterilized bottle. The bottle then fully

wrap with aluminium foil, to fully cover the bottle to avoid from light. Next, 1000 µl of 2-mercaptoethanol-β-mercapto was added into the fully covered bottle . Lastly, sdH_2O was added until it reach 500 ml. The bottle was labeled accordingly and stored on chemical working bench.

3.2.2 Preparation of tissue for total genomic DNA extraction.

Firstly, glove was used to take the sample from -80^o C freezer as precaution step to avoid hand stick to the ice. Then, the samples were taken out carefully. The frozen tissues of samples was thawed in the sink with running tap water then was washed using distilled water to remove any foreign particles. At the moment, the surgical blades, surgical blade-holder, and labeled sample were prepared. Then, the tissue samples was cut-sliced from each samples followed by storage in the properly labeled tube. Each sample was stored in separate tube. Different blade was used for each sample. The obtained in-tube-samples were stored in -20^o C freezer for the next step of DNA extraction.

3.2.3 Total genomic DNA extraction using modified CTAB method (Doyle & Doyle, 1987)

Total genomic DNA of each species of Cyprinids sample was extracted using the modified Cetyl-trimethyl Ammonium Bromide (CTAB) method (Doyle & Doyle, 1987) in the presence of the protenese K. For the solid tissue sample, appropriate amount was minced with a few drops of CTAB. The minced samples were placed into 15 ml appendorf tube, which later added with 700 µl of 2X CTAB buffer. Then 5 µl of Proteinase K was be added into the tube before it was incubated in the water bath (Protech, Model-903) at 65^o C. The tube was

incubated until the tissue samples dissolve completely. A total of 700 µl of chloroform-isoamyl alcohol (CIA) was added into the tube. Consequently, the tube was vortexes using Gilson® GVLab for 1-2 minutes to mix the solution. Later, tube was centrifuged at 13000 revolutions per minute (rpm) for 10 minutes using Hitachi, himac CF15RX, High-Speed Micro Centrifuge. Three layers of mixture was observed in the tube after centrifugation process, but only upper layer of the aqueous phase was taken out slowly using micropipette and transferred into a new tube. After that 500 µl of 100% ethanol (EtOH) was added into the tube and the tube was inverted upside down slowly to mix the mixture. Later the tube was stored overnight in the freezer at -20^{o}C before centrifugation at 13000 rpm for 15 minutes. The absolute EtOH was poured out and 500 µl of cold 70% of EtOH was added into the tube. After that, 25 µl of 3M sodium chloride (NaCl) solution was added into the tube and the mixture was mixed. Then, the samples were centrifuged again at 13000 rpm for 15 minutes. After that, the excess solution was poured out and visible DNA pellet was observed. After that, the pelleted DNA was re-dissolved in 50 µl of sterilized distilled water and was stored into the freezer at -20^{o}C for further analysis. This procedure was repeated for all samples.

3.3 Agarose gel electroporosis

Firstly, agarose gel was prepared by weighing approximately 0.5 g of agarose powder using the electronic balance (AR3130 Adventure™, Ohaus Corp), then put into the 250 ml beaker. After that, 50 ml of 1X TBE (Tris-borate-EDTA) buffer was added. Next, the mixture was heated in the microwave for 2 minutes at medium high heat. After that, the flask was swirled to ensure that the agarose powder completely dissolved. Then, the solution was poured into the contaminated beaker. Later, two drops of ethidium bromide, (EtBr) was added into the mixture and the solution was swirled to mix it well. At the moment, comb was placed inside the gel